TAS2R Activation Promotes Airway Smooth Muscle Relaxation Despite β2-Adrenergic Receptor Tachyphylaxis

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Running Head: Efficacy of a bitter tastant chloroquine in β2AR tachyphylaxis

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Recently, bitter taste receptors (TAS2Rs) were found in the lung, and act to relax airway smooth muscle (ASM) via \([Ca^{2+}]_i\) signaling generated from restricted phospholipase-C activation. As potential therapy, TAS2R agonists could be add-on treatment when patients fail to achieve adequate bronchodilation with chronic \(\beta\)-agonists. The \(\beta_2\)-adrenergic receptor (\(\beta_2\)AR) of ASM undergoes extensive functional desensitization. It remains unknown whether this desensitization affects TAS2R function, by crosstalk at the receptors or distal common components in the relaxation machinery. We studied intracellular signaling, and cell mechanics using isolated human ASM, mouse tracheal responses, and human bronchial responses to characterize TAS2R relaxation in the context of \(\beta_2\)AR desensitization. In isolated human ASM, magnetic twisting cytometry revealed >90% loss of isoproterenol-promoted decrease in cell stiffness after 18 h exposure to albuterol. Under these same conditions of \(\beta_2\)AR desensitization, the TAS2R agonist chloroquine relaxation response was unaffected. TAS2R-mediated stimulation of \([Ca^{2+}]_i\) in human ASM was unaltered by albuterol pretreatment, in contrast to cAMP signaling which was desensitized by >90%. In mouse trachea, \(\beta_2\)AR desensitization by \(\beta\)-agonist amounted to 92±6.0% \((P<0.001)\), while under these same conditions, TAS2R desensitization was not significant (11±3.5%). In human lung slices, chronic \(\beta\)-agonist exposure culminated in 64±5.7% \((P<0.001)\) desensitization of \(\beta_2\)AR-mediated dilation of carbachol-constricted airways that was reversed by chloroquine. We conclude that there is no evidence for physiologically relevant cross-desensitization of TAS2R-mediated ASM relaxation from chronic \(\beta\)-agonist treatment. These findings portend a favorable therapeutic profile for TAS2R agonists for the treatment of bronchospasm in asthma or chronic obstructive lung disease.

**Key Words:** Asthma, Airway Smooth Muscle, \(\beta_2\)-Adrenergic Receptor Desensitization, Chloroquine, and Bitter Taste Receptor
INTRODUCTION

β-agonists are routinely utilized for the treatment of asthma, for acute therapy for bronchospasm and for preventative or maintenance therapy when chronically administered. These agents relieve airflow obstruction by activating β2-adrenergic receptors (β2ARs) which are G protein-coupled receptors (GPCRs) expressed on airway epithelial and airway smooth muscle (ASM) cells (6, 44). β2ARs signal to Gαs which activates adenylyl cyclase and thereby increasing intracellular cAMP and activating protein kinase A (PKA) (7, 16). PKA phosphorylation of myosin light chain kinase and related proteins results in ASM relaxation and bronchodilation (2, 24, 42). Human studies have shown that chronic administration of standard doses of β-agonist to subjects decreases β2AR expression and agonist-stimulated generation of cAMP in circulating mononuclear cells (9) and in airway epithelial cells and macrophages obtained by bronchoalveolar lavage (43). In the treatment of obstructive lung disease, chronic β-agonist use has been associated with tachyphylaxis (loss of clinical efficacy) (15, 29). In addition, increased bronchial hyperreactivity (11, 25) and adverse affects including death (8, 20, 39, 40) have been observed in various clinical trials of chronic β-agonists. It should be noted, though, that none of these effects have been definitively linked to β-agonist evoked regulation in ASM, although β2AR desensitization seems to be the most likely of these events to be mediated by such tachyphylaxis (19, 27).

For GPCRs, agonist-promoted desensitization is defined as a loss of function during persistent agonist exposure. When function is defined as coupling of β2AR to Gαs with activation of adenylyl cyclase, β2AR desensitization is often quantified in terms of generation of the second messenger cAMP. However, in a broader context relevant to bronchodilation, desensitization can be defined as a loss of physiologic function, which is a failure to relax ASM. In this regard,
when pathways from different classes of receptors ultimately converge on the same physiologic function, desensitization can occur at points distant from the initial agonist-receptor coupling event. Furthermore, desensitization can occur via crosstalk between two pharmacologically distinct GPCRs (28, 31). Such interactions can take place via a number of interactions, including second messenger promoted kinase activation, such as PKA and protein kinase C (27).

Using RNA microarrays, the complement of GPCRs on human airway smooth muscle has been shown to be much greater than previously recognized (22), and has led to consideration of other receptors whose activation might lead to relaxation. One such class of receptors is the bitter taste receptors (TAS2Rs), some of which are expressed at levels greater than β2AR (18). TAS2Rs promote relaxation via specialized [Ca\textsuperscript{2+}]\textsubscript{i} signaling leading to membrane hyperpolarization via the large conductance calcium-dependent K\textsuperscript{+} channel (BK\textsubscript{Ca}) (18) and potentially other mechanisms (5). In isolated human bronchi, TAS2R agonists mediated relaxation appears to be equal to or somewhat greater than that of the full β-agonist isoproterenol (17, 18). Given the large number of known bitter tastants (35), these findings have given rise to the notion that these agents might be utilized for the treatment of obstructive lung disease (18). Of particular interest might be bitter tastant use when β-agonist treatment has failed, such as under conditions of tachyphylaxis. β2AR and TAS2R relax ASM by different mechanisms, but as introduced earlier, since they converge on a final common physiological function, there is the potential for the muscle that has become desensitized to the relaxation effects of β-agonists to be poorly responsive as well to TAS2R agonists. Here, we ascertain this potential using isolated human ASM mechanics and intracellular signaling in vitro, and intact human and mouse airways ex vivo, under conditions of β-agonist tachyphylaxis.
MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma (St. Louis, MO) with the exception of Dulbecco’s modified Eagles’s medium (DMEM)-Ham’s F-12 (1:1) which was purchased from Gibco (Grand Island, NY). The synthetic arginine-glycine-aspartic acid (RGD) containing peptide was purchased from American Peptide Company (Sunnyvale, CA). Albuterol, isoproterenol, salmeterol, acetylcholine, carbachol and methacholine were reconstituted in either sterile distilled water or DMSO, frozen in aliquots, and diluted appropriately in serum-free media on the day of use.

ASM cell culture and characterization. Human bronchi were obtained from lungs unsuitable for transplantation in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Human ASM cells were prepared from these bronchi as described previously (14), or were obtained from the 4th and 5th order bronchi of surgical lobectomies and pneumonectomies performed for malignancy. Cells were grown until confluence at 37°C in humidified air containing 5% CO₂ and passaged with 0.25% trypsin-0.02% EDTA solution every 10-14 days. ASM cells in culture were elongated and spindle shaped, grew with the typical hill-and-valley appearance, and showed positive staining for smooth muscle specific α-actin. In this study, we used cells in passages 3-7, as these cells retain native contractile protein expression (37). Unless otherwise specified, serum-deprived post-confluent cells were plated at 30,000 cells/cm² on plastic wells (96-well Removawell, Immunlon II: Dynetech) previously coated with type I collagen (Vitrogen 100; Cohesion, Palo Alto, CA) at 500 ng/cm². Cells were maintained in serum-free media for 24 h at 37°C in humidified air.
containing 5% CO₂. These conditions have been optimized for seeding cultured cells on collagen matrix and for assessing their mechanical properties (3, 4, 18).

**Magnetic twisting cytometry (MTC).** Dynamic changes in stiffness were measured as an indicator of contraction and relaxation of isolated human ASM cells, using MTC as described by us in detail elsewhere (3, 18). In brief, RGD-coated ferrimagnetic microbeads (4.5 μm in diameter) bound to the surface of adherent human ASM cells were magnetized horizontally and then twisted in a vertically aligned homogenous magnetic field that was varying sinusoidally in time. This sinusoidal twisting magnetic field caused both a rotation and a pivoting displacement of the bead: as the bead moves, the cell develops internal stresses which in turn resist bead motions (23). Lateral bead displacements in response to the resulting oscillatory torque were detected with a spatial resolution of ~5 nm, and the ratio of specific torque to bead displacements was computed and expressed here as the cell stiffness in units of Pascal per nm (Pa/nm).

In this study, adherent human ASM cells were treated for 18 h with or without 1 μM albuterol. In both albuterol-treated and -untreated ASM, methacholine increased cell stiffness in a dose-dependent manner, with a maximal response at 10 μM which was not different between treated or untreated cells (data not shown). Unless otherwise stated, ASM cells were contracted with 10 μM methacholine which was maintained during addition of multiple doses of isoproterenol or chloroquine. For each individual human ASM cell, changes in stiffness in response to isoproterenol or chloroquine were normalized to its respective methacholine-contracted stiffness.
**[Ca\textsuperscript{2+}]\textsubscript{i} and cAMP measurements.** For detecting changes in [Ca\textsuperscript{2+}]\textsubscript{i}, adherent cells in 96-well plates were loaded with Fluo-4 AM (BD Biosciences) and probenecid for 1 h at 37°C. Receptor agonists were added by an automated pipetting system in triplicate, and the 525 nm signals were generated by excitation at 485 nm using a Flex Station II (Molecular Devices). Data were acquired every 1.5 s for 1 min. Unless otherwise stated, studies were performed in media containing 1.5 mM calcium. For cAMP measurements, human ASM cells were plated in 96-well plates and detected using a fluorescence-based assay (CatchPoint, Molecular Devices).

**Ex vivo intact mouse airway physiology.** All mouse studies were approved by the Animal Care and Use Committee of the University of Maryland, School of Medicine. As described previously (18), we excised 5-mm sections of trachea from FVB/N mice (Taconic) and studied them in an isometric myograph system. In brief, tracheal rings fitted between a fixed wire and a transducer-coupled wire were maintained in Krebs buffer saturated with 95% O\textsubscript{2}, 5% CO\textsubscript{2} at 37°C. A passive tension of 5 mN was applied for each ring for a baseline. For relaxation studies, rings were contracted with acetylcholine (0.1 mM), which was maintained during addition of isoproterenol (10 μM) or chloroquine (1 mM). Then, rings were washed, removed from tension, and incubated with isoproterenol (10 μM) in Krebs buffer with 0.1 mM ascorbic acid for 18 h at 37°C in a 5% CO\textsubscript{2}, 95% air atmosphere. Rings were then washed, contracted with acetylcholine, and relaxed again with isoproterenol or chloroquine as indicated above. Desensitization was calculated as the % loss of relaxation of the post-18 h isoproterenol exposed rings compared to the pre-exposed rings (control), with each ring acting as its own control.
**Human precision-cut lung slice (PCLS) preparation and airway function.** Healthy human lungs were received from the National Disease Research Interchange (NDRI), and the PCLs were prepared as previously described (12, 13). Human PCLs (approximately 750 μm thick) were then maintained in 24-well plates with continuous agitation in 0.5 ml culture medium (DMEM:F12) supplemented with 15 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml of amphotericin B at 37°C in humidified air containing 5% CO₂. After at least 48 h in culture, lung slices were washed and incubated for 18 h in the absence or presence of 50 nM salmeterol or 1.0 μM albuterol. Slices were then washed 4 times. After collecting a baseline quantitative microscopic image (under conditions of 0% contraction), human PCLs were constricted with 1 μM carbachol and then treated sequentially with 1 μM isoproterenol and 50 μM chloroquine. For each treatment, airway images were collected after 15-20 min and the lumenal area measured using a program written within Image J software. Bronchodilation was calculated as the % reversal of the carbachol-induced reduction in airway cross sectional area.

**Statistical analysis.** For MTC, cAMP, and mouse trachea studies, we used Student’s t-test, the Analysis of Variance (ANOVA) with adjusting for multiple comparisons by applying the Bonferroni’s methods, or nested design analysis to control any random effect caused by repeated measurements of multiple cells (MTC) in the same subject. To satisfy the normal distribution assumptions associated with ANOVA, cell stiffness data were converted to log scale prior to analyses. For PCLs, analysis was by ANOVA using the Holm-Sidak method for pairwise multiple comparisons. All analyses were performed in SAS V.9.2 (SAS Institute Inc., Cary, NC), and the 2-sided P-values less than 0.05 were considered significant.
RESULTS

Chloroquine is highly efficacious in decreasing the stiffness of human ASM cells. We established primary human ASM cell cultures from five separate lungs and, using MTC, measured their mechanical responses to the β2AR agonist isoproterenol and a selective TAS2R agonist chloroquine. For these studies, ASM cells were first contracted for 5 min with methacholine (10 μM) and then relaxed with increasing doses of either isoproterenol or chloroquine. In methacholine-contracted ASM cells, maximal decreases in stiffness occurred with 10 μM isoproterenol and 1 mM chloroquine, respectively, consistent with our previously reported responses under other conditions (18). Human ASM cells isolated from the lung donors showed some degree of between-cell and -donor variation in isoproterenol- and chloroquine-induced stiffness responses (Fig. 1a,b). Nevertheless, the maximal reduction in cell stiffness induced by chloroquine was greater in magnitude than isoproterenol, differences ranging from ~7 to 44% among individual lung donors (Fig. 2a). Applying nested design analysis, we found statistical differences between the effects of chloroquine and isoproterenol on cell stiffness as early as 6 s ($P<0.0001$ vs isoproterenol) upon and throughout drug stimulation, with the maximal differences at time 87 s ($P<0.00005$ vs isoproterenol). In addition, there were significant differences ($P<0.00001$) in the rate of stiffness decreases between isoproterenol (-0.00018 per s) and chloroquine (-0.00251 per s). These findings demonstrate that TAS2R activation by chloroquine has a faster onset of action and greater efficacy than the full β2-agonist isoproterenol in relaxing methacholine-contracted human ASM.

Prolonged exposure to albuterol induces β2-AR desensitization in isolated human ASM. To induce β2AR desensitization (13), we treated isolated human ASM cells for 18 h with 1 μM
albuterol. Subsequently, cells were washed, the response to methacholine ascertained, and then
their mechanical responses to the β₂AR agonist isoproterenol and a selective TAS2R agonist
chloroquine were measured. Albuterol-treated and -untreated cells exhibited differential cellular
responses to isoproterenol (Fig. 1a,c). In albuterol-untreated cells, isoproterenol (10 μM) caused
stiffness decreases that ranged from ~8 to 21% reduction from the methacholine-contracted state
(Fig. 2a). 10 μM of this full β₂-agonist isoproterenol had little effect on the stiffness of
albuterol-treated cells contracted with methacholine, even at higher doses of isoproterenol (data
not shown). These findings are consistent with previous findings in human lung slices (13), and
demonstrate a direct effect, independent of the epithelium, of long-term β-agonist exposure on
receptor desensitization in isolated human ASM cells. As shown in Fig. 1, this extensive β₂AR
desensitization by β-agonist was observed with cells from each of the five donors.

β₂AR desensitized in human ASM cells are fully responsive to chloroquine. Under these same
conditions that essentially eliminated the effectiveness of a full β₂AR agonist isoproterenol, we
found that a selective TAS2R agonist chloroquine (1 mM) substantially decreased stiffness of
albuterol-treated cells from each donor (Fig. 1b,d and Fig. 2). In fact, chloroquine evoked
nearly identical degrees of reduction in stiffness of both albuterol-treated and -untreated human
ASM cells (Fig. 3), amounting to 39±3.9% and 40±7.2% maximal reduction from their
respective methacholine-contracted state (P=NS). These results indicate a lack of
physiologically relevant cross-desensitization between the β₂AR pathway and the TAS2R
pathway. To further confirm this notion, human ASM in culture were exposed to carrier
(ascorbic acid) or β-agonist for 18 h, washed, and cAMP (the β-agonist response) or [Ca^{2+}]_i (the
TAS2R response) measured with exposure to isoproterenol or chloroquine, respectively. As
shown in Fig. 4a, long-term β-agonist exposure markedly desensitized (>90%) the isoproterenol-stimulated cAMP response, but did not alter chloroquine-mediated [Ca^{2+}]_i release (Fig. 4b).

**Chloroquine responsiveness during β\textsubscript{2}AR desensitization in intact airways.** We next examined the efficacy of chloroquine in relaxing ASM of intact mouse airways, under conditions of β\textsubscript{2}AR desensitization. For each control tissue pre-contracted with a sub-maximal dose of acetylcholine, both isoproterenol and chloroquine decreased the force generating capacity of mouse trachealis (Fig. 5a,b). Consistent with our previous report (18), however, chloroquine promoted reduction in ASM tension was greater in magnitude than that of isoproterenol in tissues that had not been pre-treated with β-agonist (Fig. 6). In tissues that were pre-treated for 18 h with isoproterenol, and then washed, acetylcholine induced tension development was similar in magnitude as in time matched, untreated tissues (data not shown). Subsequent isoproterenol treatment, however, showed a significant decrease in relaxation (from 59±5.2% to 5.3±4.3%, \(P<0.001\)) representing a 92±6.0% desensitization of the response (Fig. 5a,c). In contrast, as with isolated ASM measurements with MTC, chloroquine markedly decreased ASM tension in tissues that had been pre-treated with β-agonist, with desensitization amounting to 11±3.5%, which was not statistically significant (Fig. 5d). Indeed, the reduction in ASM tension with chloroquine was indistinguishable from that of time-matched, non-β-agonist treated airways (Fig. 6).

Based on our established intact human airways model demonstrating decreased sensitivity to isoproterenol-mediated bronchodilation following chronic exposure to β-agonists (12, 13) and, compelling evidence for increased bronchial hyperresponsiveness with regular use of long-acting β-agonist salmeterol (11, 12, 45, 46), we incubated human precision-cut lung slices (PCLSs) for 18 h in the absence (control) or presence of 50 nM salmeterol. This dosage of salmeterol is as
equieffective in bronchodilating PCLSs, and in desensitizing $\beta_2$AR when chronically administered, as 1 μM albuterol (12). For each control PCLS pre-contracted with a sub-maximal dose of carbachol, both isoproterenol and subsequent chloroquine increased the luminal area of these airways, amounting to 65±4.2% and 75±7.8% bronchodilation ($P$=NS), respectively, at doses that were 1/10th and 1/20th of those used for mouse tracheal rings (Fig. 7). In human PCLSs that were pre-treated for 18 h with salmeterol, however, isoproterenol treatment showed 64±5.7% desensitization of airway lumen dilation ($P<0.001$ vs control isoproterenol) and subsequent chloroquine treatment markedly bronchodilated these carbachol-constricted airways (Fig. 7). Chloroquine also effectively rescued desensitization in carbachol-constricted airways (73±6.7% bronchodilation; mean ± SE, n=4) that were chronically exposed to albuterol. Taken together, our findings in intact mouse and human airways physiology *ex vivo* and isolated human ASM mechanics and intracellular signaling *in vitro* demonstrate a consistent $\beta_2$AR desensitization with chronic $\beta$-agonist exposure in airway smooth muscle regardless of the model. Despite such $\beta_2$AR desensitization, however, we show no physiologically relevant cross-desensitization between the $\beta_2$AR pathway and the TAS2R pathway, and that TAS2R activation is highly efficacious in relaxing ASM and bronchodilating the airways under conditions of $\beta$-agonist tachyphylaxis.
DISCUSSION

We have contrasted signaling and mechanical responses of primary human ASM cells, and airway relaxation responses in human and mouse bronchi to a selective TAS2R agonist chloroquine in the context of the β-agonist-desensitized state. These studies were prompted by the recent finding that TAS2Rs are expressed on ASM, and act to markedly relax ASM and bronchodilate the airways. These studies have brought to the forefront the potential for a new class of direct bronchodilators acting at TAS2Rs. These agents might be used in patients with asthma or chronic obstructive lung disease who have not achieved adequate control on chronic β-agonists, “indirect” bronchodilators (such as M3-receptor antagonists) and other agents. As introduced earlier, chronic β-agonist activation of β2AR is associated with clinical deterioration which may be due to β2AR desensitization. Such desensitization, particularly when defined by the end-organ response, can act across multiple nodal points including at the receptor, G-protein, the “initial” effector (such as adenylyl cyclase) and more distal effectors. Crosstalk has been shown among multiple GPCRs, even those with disparate structures and ligands (26, 30-33, 36). We thus felt it necessary to explore the potential for chronic β-agonist activation of β2AR on ASM to cross-desensitize TAS2R responses. Studies were carried out measuring intracellular signaling, isolated ASM mechanics, and intact mouse and human airways.

In studies of isolated ASM mechanics with MTC, chloroquine had a more rapid onset and greater efficacy than isoproterenol in decreasing stiffness of an individual human ASM cell from each lung donor. As previously shown in human lung slices (13), here we have also demonstrated β2AR desensitization in isolated human ASM cells with long-term exposure to β-agonists. Whereas this exposure inhibited generation of cAMP and completely abrogated the ability of an individual human ASM cell to decrease stiffness in response to isoproterenol, such
receptor desensitization did not affect the efficacy of chloroquine. Chloroquine evoked intracellular calcium release and decreased stiffness of isolated human ASM cells, even with β-agonist promoted β₂AR desensitization. Scaling up to the level of intact airways *ex vivo*, chloroquine markedly inhibited force generating capacity of mouse trachealis as well as effectively bronchodilated human airways that exhibited tolerance to β-agonists. These findings further confirm that TAS2R activation utilizes a novel GPCR pathway that is distinct from Gₙ/Go-mediated activation of downstream targets and suggest limited deleterious cross-talk with β-agonists in promoting ASM relaxation.

Desensitization of GPCR signaling is frequently encountered in both *in vitro* and *in vivo* systems. Some receptors display little or no detectable desensitization (21) while others, such as the β₂AR, undergo a marked decrease in function, as exhibited by measurements of the second messenger cAMP (*Fig. 4a*) or the end-organ response (*Figs. 2, 6, 7*). We have previously shown only a modest degree of desensitization (20-30%) of TAS2R function with bitter tastant exposure in human ASM and monkey bronchus (38). At issue for the current studies is whether the markedly desensitized relaxation response evoked by prolonged β-agonist treatment affects the relaxation response to TAS2R activation. As discussed earlier, ample evidence suggests that interaction can occur between different GPCRs at multiple points within the signaling cascade from initial receptor activation and coupling to G-protein, to further downstream components. We show here no evidence for such interaction between chronic β-agonist evoked β₂AR desensitization and TAS2R function in ASM, as determined by intracellular signaling measurements, single cell mechanics, and mouse and human airway relaxation responses. Importantly, our studies, derived from tissues retaining much of the native architecture of small
human airways, provide further support for the idea that parallel pathways for bronchodilation exist that could be used therapeutically.

TAS2Rs belong to a family of GPCRs that is composed of 25 members (1, 10, 34) and, in human ASM, three dominant TAS2Rs (subtypes 10, 14, 31) are detected at the mRNA and functional levels (18). TAS2Rs function in airways is mediated by $[\text{Ca}^{2+}]_i$ release, but nevertheless, leads to ASM relaxation and bronchodilation. This is based on a concordance of relaxation potency and efficacy with measured $[\text{Ca}^{2+}]_i$, release, loss of TAS2R-mediated relaxation when $[\text{Ca}^{2+}]_i$ release was blocked, and stimulation of spatially and temporally distinct $[\text{Ca}^{2+}]_i$ events evoked by TAS2R agonists in intact ASM (18). In that study, we considered that the large conductance calcium-dependent $K^+$ (BK$_{Ca}$) channels were activated, and thus played some role in the relaxation response, based on a sensitivity of TAS2R-mediated relaxation of mouse airways, and individual ASM cells, to the BK$_{Ca}$-channel inhibitor iberiotoxin (IbTX).

This remarkable degree of ASM relaxation and its unique mechanism of action has lead to the consideration of developing TAS2R agonists for treating obstructive lung disease. TAS2Rs are also expressed on airway epithelial cells, and upon activation increase ciliary beat frequency (41), which may also have a therapeutic effect by increasing mucous clearance. We have previously shown, using submaximal doses, that the acute relaxation effects of $\beta$-agonist and TAS2R agonist are additive (18). Thus concomitant use of both agents can be envisioned, providing two direct bronchodilators which act via different mechanisms. We now know that even under conditions of marked $\beta_2$AR desensitization of the relaxation response, TAS2R agonists maintain full efficacy, which is a favorable property in consideration of taking forward TAS2R agonists as novel treatment options.
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DISCLOSURES

The authors declare no competing financial interests.
AUTHOR CONTRIBUTIONS

S.S.A, performed single-cell mechanics, data analysis and interpretation, and wrote the manuscript; W.C.H.W., *ex vivo* intact mouse airway physiology, intracellular calcium and cAMP measurements, data analysis and manuscript preparation; C.J.K.W., isolated human ASM cells, prepared human lung slices, and performed airway function; K.A., statistical analysis; D.Y.L., single-cell mechanics and data analysis; R.C.K., performed intact human PCLS responses, data analysis and interpretation, and manuscript preparation; R.A.P., established primary human ASM cell culture and intact human lung slices, data analysis and interpretation, and manuscript preparation; S.B.L., directed all studies, data analysis and interpretation, and is the primary author of the manuscript.
**FIGURE LEGENDS**

**Figure 1.** Dynamic changes of cell stiffness in response to a full β2AR agonist isoproterenol and a selective TAS2R agonist chloroquine in human ASM as assessed by Magnetic Twisting Cytometry. Primary human ASM cell cultures were established from 5 separate lung donors. Isolated human ASM cells were (a, b) untreated or (c, d) treated for 18 h with 1 μM albuterol. Cells were contracted with 10 μM methacholine (MCh), and then relaxed with (a, c) 10 μM isoproterenol (Iso) or (b, d) 1 mM chloroquine (Chloro). For each cell, changes in stiffness in response to Iso or Chloro were normalized to its respective MCh-contracted stiffness. Data are presented as median (a, n = 53 to 266; b, n = 62 to 281; c, n = 58 to 248; d, n = 70 to 233 individual cell measurements).

**Figure 2.** Maximum stiffness reduction of methacholine-contracted human ASM induced by isoproterenol and chloroquine in (a) untreated and (b) albuterol exposed cells. Data are presented as mean ± SE (a, n = 53 to 281; b, n = 58 to 248 individual cell measurements). *, P < 0.01; #, P < 0.00005.

**Figure 3.** Dynamic changes of cell stiffness in response to 1 mM chloroquine in untreated and albuterol-exposed human ASM. To control random effects due to multiple cell measurements from the same donor, the nested regression was used for group comparisons. Data are presented as mean ± SE (n = 775 to 859 individual cell measurements from 5 lung donors).
Figure 4. $\beta_2$AR desensitization and intracellular signaling in isolated human ASM cells. (a) Cultured primary human ASM cells were incubated for 18 h with ascorbic acid (AA) or $\beta$-agonist (Iso), and cAMP measured by CatchPoint assay (Molecular Devices). Isoproterenol mediated accumulation of cAMP (15 min) is abrogated in human ASM cells that were pre-treated for 18 h with $\beta$-agonist, but not in untreated cells (mean $\pm$ SE, $n = 4$ experiments). *, $P < 0.01$. (b) Human ASM cells were loaded with Fluo-4 AM, and $[Ca^{2+}]_i$ release evoked by TAS2R activation was measured. Arrow indicates the time of chloroquine addition. $[Ca^{2+}]_i$ transients curves to 1 mM chloroquine is unabated in human ASM cells that were pre-treated for 18 h with $\beta$-agonist. Results shown are from a single representative experiment of 3 performed.

Figure 5. TAS2R activation evokes ASM relaxation under conditions of $\beta_2$AR desensitization. Intact mouse trachealis were studied in the absence (Control; a,b) or after 18 h of exposure to the $\beta$-agonist isoproterenol (Post-Iso treatment; c,d). Rings were contracted with 0.1 mM acetylcholine (Ach), and relaxed with 10 $\mu$M isoproterenol (Iso1, Iso2) (a,c). After each Iso treatment, the ring was washed and then rechallenged with the same dose of Ach, followed by 1 mM chloroquine (Chloro1, Chloro2) addition (b,d). Ach was maintained in the bath when Iso and Chloro were added. Results shown are from a single representative experiment of at least 4 performed.

Figure 6. TAS2R activation is highly efficacious in relaxing ASM under conditions of $\beta_2$AR desensitization. As described above in Fig. 4, intact mouse trachealis were studied before...
(Control) or after treatment with the β-agonist isoproterenol (Post-Iso treatment). Subsequent Iso and Chloro responses were measured in rings contracted with Ach. Data are expressed as mean ± SE (n = 4 experiments). *, P < 0.01.

**Figure 7.** TAS2R activation dilates intact human small airways under conditions of β_{2}AR desensitization. Human PCLSs were incubated for 18 h in the absence (Control) or presence of long-acting β-agonist salmeterol (Post-Sal treatment), washed, and constricted with carbachol. Carbachol-constricted airways were treated sequentially with 1 μM Iso and 50 μM Chloro. Data are presented as mean ± SE (obtained using 102 PCLSs derived from 4 donors). *, P < 0.005.
REFERENCES


Fig 1
A  Untreated

B  Albuterol Exposed

Fig 2
Fig 3

Cell Stiffness (ratio of MCh stimulation)

Time (s)

Time points:
- Untreated
- Albuterol Exposed

Chloro
Fig 4
Fig 5
Fig 6

Comparison of % Relaxation between Control and Post-Iso treatment:
- Iso 1
- Chloro 1
- Iso 2
- Chloro 2

Significance indicated by asterisk (*)
N.S. denotes non-significant difference.
Fig 7